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Intestinal microbiota of striped catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878) fed on dietary nucleotide

Maryam YAGHOBI[1], Fatemeh PAYKAN HEYRATI[*1], Mostafa AKHLAGHI[2], Salar DORAFSHAN[1], Nemat MAHMOUDI[3]

[1]*Department of Natural Resources, Isfahan University of Technology, Isfahan, 84156-83111, Iran.*
[2]*School of Veterinary Medicine, Shiraz University, Shiraz, Iran.*
[3]*Department of Fisheries, Faculty of Natural Resources and Marine Sciences, Tarbiat Modares University, Noor, Mazandaran, Iran.*

Email: fheyrati@cc.iut.ac.ir

Abstract: The aim of this study was to assess the effects of nucleotide on intestinal microbiota of striped catfish, *Pangasianodon hypophthalmus*. Different levels of the NT (0, 0.25. 0.5, 0.75 and 1 % weight per weight, WW^{-1}) were added to basal diet and then randomly allocated to triplicate groups of fish with average initial weight (1.52±0.11 g) for 10 weeks. At the end of the experiment, at least 10 individual fish from each treatment were used to study quantitative and qualitative analyses of bacterial flora of intestine. The bacterial flora was identified to species level where possible. Total viable bacterial counts in the intestine of the catfish ranged from $4.33\pm0.49\times10^4$, $5.98\pm0.82\times10^4$, $6.44\pm1.09\times10^4$, $5.54\pm0.64\times10^4$ and $2.65\pm0.46\times10^4$ colony forming units (cfu/g) for groups treated with 0-1% NT respectively without any significant difference among groups ($P>0.05$). Gram negative rod shaped bacteria were dominant in all groups. Altogether 16 bacterial species of 12 genera were identified in total populations. Most of the bacterial species were common among all groups. *Proteus mirabilis, Serratia liquefaciens, Bacillus cereus* only were observed in NT treated fish while *Alkaligenes faecalis* only existed in the control group. *Corynebacterium urealyticum* and *Staphylococcus aureus* were identified in some fish belong to control-0.25 NT and control 1-NT groups respectively. The results of this study demonstrate that although the total bacterial count number did not affected by dietary NT but some qualitative changes were clearly observed.

Keywords: Bacterial flora, Bacteriological analysis Dietary nucleotide, Striped catfish, Siluriformes.

Introduction

Striped catfish, *Pangasianodon hypophthalmus* (Sauvage 1878) (Siluriformes: Pangasiidae) is an endangered (IUCN 2014) native freshwater fish species of inland water of the south-east Asia mainly Thailand, Viet Nam, Laos and Cambodia. This species has been introduced to different countries such as Singapore, the Philippines, the USA as well as Iran as a cultivated food fish or an ornamental hobby species. Some major threats such as over exploitation, habitat degradation in water quality and flow cause decreasing native and wild population (Vidthayanon & Hogan 2011). Despite declining wild population, this species has a key role in aquaculture industry in many countries such as Thailand and Viet Nam.

Fish cultivation practices under intensive condition such as grading, handling, transportation as well as poor water quality can reduce fish welfare and increase the risk of fish disease. Nowadays, different practices could be used to improve aquatic welfare, among them food additives use in commercial scale.

Nucleotides are one of the most important food components which frequently found in intracellular area of different organisms. These chemicals have varieties of physiological and biochemical roles in organisms such as fish (Li & Gatlin III 2006), and shellfish (Shankar et al. 2012). Although, most animals can synthesize NT, the recent research showed that dietary nucleotide supplementation could improve growth performance (Burrells et al. 2001b; Borda et al. 2003; Li & Gatlin III 2007), immune gene expression and immune response (Low et al. 2003; Tahmasebi-Kohyani et al. 2011), disease resistance (Burrells et al. 2001a; Shankar et al. 2012) and intestinal morphology (Burrells et al. 2001; Cheng 2011; Oujifard et al. 2012). The influence of intestine flora on the host has great interest in fish culture (Lavens & Sorgeloos 2000). Different parameters such as age, nutritional status and aquatic digestive tract structure may affect the bacterial microflora (Al-Harbi & Uddin 2004). It is also some evidence showing the effects of environmental condition on gut microflora (Ringo et al. 2006). Many studies showed that different food additives such as probiotics could affect intestinal microbiota in different fish species such as stellate sturgeon, *Acipenser stellatus* (Akrami et al. 2013) and Caspian roach, *Rutilus rutilus* (Hoseinifar et al. 2013). However, to the best of our knowledge there is no information regarding the effects of dietary NT on intestinal microbiota in fish. Hence, the present study was carried out to study any probable effects of dietary NT on intestine microbiota in the striped catfish, *Pangasianodon hypophthalmus* under laboratory condition.

Materials and methods

Fish and feeding trail: 800 striped catfish fry (0.7±0.05g) were bought from a commercial supplier, Sepanta Mahianab Aria Pars, Isfahan, Iran. The fish were acclimatized to the laboratory conditions for two weeks before starting the experiment. At the beginning of the experiment, the fish were weighted (1.52±0.11g) and then randomly stocked into 15 150-L aquaria (50 fish aquarium^{-1}) in triplicate per dietary treatment. The water level in aquaria was stable and about 50% of the water body was changed every two days to maintain the water quality parameters. Water temperature, pH, and dissolved oxygen were 30±2.0°C, 6.5±0.2 and 5mgL^{-1}, respectively. Fish were fed about 5% of body weights, three times a day at 10:00, 13:00 and 16:00 for 70 days. A practical diet formulated based on fish meal, soybean oil, soybean meal, corn, corn gluten and fish oil containing 39% crude protein, 14% fat, 21.7% carbohydrate, 3% of fibre and less than 10% moisture proposed by National Research Council (NRC 1973) was supplemented with the commercial nucleotide mixture "Optimun" (Chemoforma, Augst, Switzerland) to give 0, 2.5, 5, 7.5 and 10g of mixed NT Kg^{-1} diet. Optimun contained inosine monophosphate (IMP), adenosine monophosphate (AMP), guanosine monophosphate (GMP) and ribonucleic acid (RNA).

Bacteriological sampling and analyses: At the end of the experiment, fish sampling were carried out for bacteriological analysis after 24 hours of starvation. At least, 10 fish (16.82±0.39g) were randomly selected from each treatment. The fish surface was sterilized with ethanol (70%) and the gut was removed. Each gastrointestinal tract was preserved in separate bag at -20±1°C for about 2 months until analysis. Frozen fish were thawed at room temperature (1 to 2hrs.) until it was soft enough to sample. The number of incidental organisms was reduced by using sterile scissors. Around 5g of intestine from sampled fish was taken aseptically, mixed and homogenized in a mortar. Samples were taken from mid-parts of the whole intestines to standardize the sampling. About 2g of homogenate was suspended to a bottle containing 25ml of sterile (121°C, 15min) 0.85% (wv-1) NaCl prepared in de-ionized water. The suspension (1mL) was serially diluted to 10-fold dilutions. Volumes (0.1mL) of the dilutions were spread onto tryptone soya agar plates (TSA, Oxoid, UK) in duplicate.

Total aerobic heterotrophic bacterial counts of the

intestine were determined by incubation of all the inoculated plates at 25°C for 48 hrs. and colony forming units (CFU) were counted with a dark-field colony counter equipped with a guide plate ruled in square centimeters. The plates having ≥ 30 to 300 colonies were used to calculate bacterial population numbers, expressed as CFU.

Isolation of bacteria: To determine the percent composition of bacteria types in the samples, bacterial colonies were divided into different groups according to colony characteristics (shape, size, elevation, structure, surface, age, color and opacity) and counted the number of colonies of each recognizable type. Generally, 3 to 5 representatives of each colony type were then streaked on TSA plate repeatedly until pure cultures were obtained. Purified cultures were inoculated onto TSA slants and kept at 4°C for stock.

Identification of bacteria: To identify the selected bacterial isolates to genus or species levels, the purified isolates were observed for cell shape, motility, flagellation, spores, encapsulation, and Gram staining. The isolates were then subjected to biochemical tests (oxidase, catalase, amylase, lipase, indole production, methyl red and Vogues Prousker tests and nitrate reduction) following the criteria described in the Bergey's Manual of Determinative Bacteriology (Holt 2000). In parallel, the commercial API 20E, strip, (bioMerieux, France) methods were also used (Buller 2004).

Statistical analysis: Statistical analysis was performed by one way ANOVA at 5% significant level. A multiple comparison test (Tukey studentised range test, TMRT) was conducted to compare the significant differences among the groups using SPSS V.19. Values are presented as mean ± standard error of mean.

Results

The effects of different dietary nucleotide levels on intestinal microbiota of striped catfish were shown in Figure 1. During the study, total count of bacteria in intestine of fish fed 1, 0.75, 0.5, 0.25% nucleotide and control group were 2.56±0.46, 5.54±0.64, 6.44±1.09, 5.98±0.82 and 4.33±0.49 (cfu/g)×10^4, respectively. There were significant differences between fish fed 0.5, 0.25% nucleotide and group with maximum nucleotide (P<0.05).

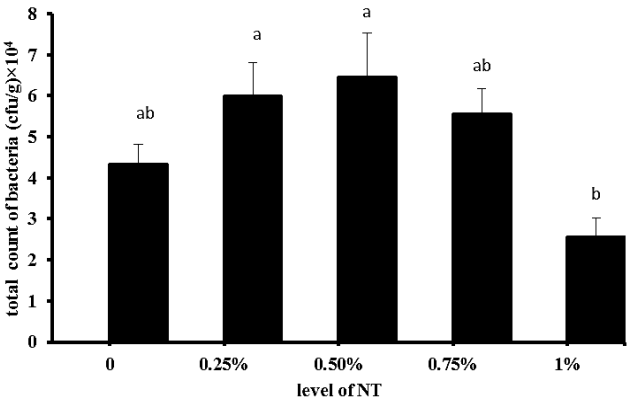

Fig.1. Effect of dietary supplemental nucleotide on intestinal microbiota of striped catfish. Data were presented as mean with standard error as error bars; Significant differences (P<0.05) between groups is indicated by unlike letters on the bars for each intestine bacteria.

Ten bacterial species were identified from 1% group (Table 1). Furthermore eight, eleven, thirteen and thirteen bacterial species were found in catfish intestine of fish fed dietary contain 0.75, 0.5, 0.25 and 0% nucleotide, respectively. Members representing three species viz. *Hafnia alvei*, *Pseudomonas fluorescence*, *Schewanella putrefacien*, were dominant in control group. *Micrococus luteus* and *P. fluorescence* were dominant bacteria in fish fed 0.25% nucleotide. *Micrococus luteus*, *P. fluorescence* and *S. putrefacien* were common to fish fed dietary contain 0.5 and 0.75% nucleotide. *Micrococus luteus* and *S. putrefacien* were most dominant bacteria in catfish fed 1% nucleotide. All the bacteria counts including *M. luteus*, *S. putriefacien* and *Aeromonas caviae* showed the increasing trend with the increase of the dietary nucleotide until 0.5%, however, no significant differences were found (P>0.05), after increasing nucleotide, bacteria number decreased. The result showed *Enterobacter aerogen* and *Bacillus subtilis aerus* in 1 and control group but other treatments did was not find in the 0.75%. There were

Table 1. Effect of dietary supplemental nucleotide on intestinal microbiota of striped catfish.

Variable (units)	0%	0.25%	0.5%	0.75%	1%
total count of bacteria (cfu/g)×10^4	4.33 ± 0.49^{ab}	5.98 ± 0.82^{a}	6.44 ± 1.09^{a}	5.54 ± 0.64^{ab}	2.56 ± 0.46^{b}
Dominant bacteria in culture*	*Proteus vulgaris, Pseudomonas fluorescence, Bacillus subtilis, Aeromonas caviae, Schewanella putrefaciens, Hafnia alvei Enterobacter aerogenes, Micrococcus luteus, Alcaligenes faecalis, Proteus mirabilis Staphylococcus aureus, Aeromonas hydrophila, Corynebacterium urealyticum*	*Micrococcus luteus, Proteus vulgaris, Pseudomonas fluorescence, Bacillus cereus, Schewanella putrefaciens Serratia liquefaciens Proteus mirabilis, Aeromonas hydrophila, Bacillus subtilis, Aeromonas caviae, Hafnia alvei, Corynebacterium urealyticum Enterobacter aerogenes*	*Micrococcus luteus, Proteus mirabilis, Pseudomonas fluorescence, Aeromonas caviae, Schewanella putrefaciens, Aeromonas hydrophila, Hafnia alvei, Bacillus subtilis, Proteus vulgaris, Bacillus cereus, Enterobacter aerogenes*	*Proteus vulgaris, Pseudomonas fluorescence, Micrococcus luteus, Aeromonas caviae, Schewanella putrefaciens, Hafnia alvei Proteus mirabilis, Serratia liquefaciens, Aeromonas hydrophila*	*Micrococcus luteus, Pseudomonas fluorescence, Schewanella putrefaciens, Enterobacter aerogenes Proteus mirabilis, Aeromonas hydrophila, Aeromonas caviae, Proteus vulgaris, Bacillus subtilis, Staphylococcus aureus*

staphylococcus not show it. *Hafnia alvei* and *Alcaligenes faecalis* only observed in 1%NT and control groups, respectively. Fish fed dietary supplemented with 0.25 and 0.75% nucleotide contained *Serratia liquefaciens* in the intestine, whereas other groups did not show it. *Bacillus cereus* was not observe in fish fed 0, 0.75 and 1%, whereas *Corynebacterium urealyticum* found in 0.25% and 0 groups.

Discussion

The alimentary tract of fishes represents an interface between the external environment and the body. Its complex polymicrobial ecology interacts with the internal and external environment and has an important influence on health and disease. The intestine is a complex multifunctional organ. In addition to digesting and absorbing feedstuff, it is critical for water and electrolyte balance, endocrine regulation of digestion, metabolism and immunity. The GI microbiota of fish is characterized by high population density, wide diversity and complexity of interactions. While all major groups of microbes are represented bacteria predominance. They are the main constituent of the gut microbiota in fish (Deven et al. 2009). Competition for nutrients or available energy can play an important role in the composition of the GI microbiota or in the culture water of aquatic species.

Many variations in morphology of the GI tract exist between various fish species. Depending on feeding habits and diet, it is generally accepted to divide fish into carnivores (eating fish and bigger invertebrates), herbivores (consuming mainly plant material), omnivores (mixed diet eaters) and detrivores (feeding largely on detritus). The type of food is important for composition and activity of the fish GI microbiota. Fishes possess specific intestinal microbiota consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria. This microbiota has been classified as autochthonous or indigenous (when they are able to colonize the host's gut epithelial surface) or as allochthonous or transient. Several studies on various fresh- and saltwater fish have demonstrated bacteria in the intestinal lumen and associated with the intestinal

epithelium. Bacteria attached to epithelial surfaces have been demonstrated in the gut of a variety fish species, and it has been suggested that this attachment is an important factor in determining whether a particular organisms colonizes in the intestinal tract. It therefore, seemed likely that the presence of consistently high numbers of beneficial bacteria was dependent on their ability to colonize the intestinal surface. The colonization of the GI tract of fish larvae starts immediately after hatching and is completed within a few hours. Colonizing bacteria can modulate expression of genes in the digestive tract, thus creating a favorable habitat for them and preventing invasion by other bacteria introduced later into the intestinal ecosystem. Some investigations have reported that bacteria present in the hatchery environment may influence the composition of GI microbiota. Using a culture-based approach, these results suggest that bacteria present in the GI tract generally seem to be those from water or the diet, and which can survive and multiply. Furthermore, larvae may ingest substantial amounts of bacteria by grazing on suspended particles and egg debris. Hence, it is tempting to suppose that egg microbiota would also affect the primary colonization of the fish larvae.

These attached (resident) bacteria are responsible for enteric bacterial antagonism and colonization resistance, since they are associated closely with the intestinal epithelium, and form a barrier, serving as the first defense to limit direct attachment or interaction of fish pathogenic bacteria to the gut mucosa. The equilibrium between species of resident bacteria provides stability in the microbial population within the same individual under normal conditions. From a microbial point of view, it is important to have stable resident intestinal microbiota as a part of the natural resistance of fish to infections.

Understanding some aspects of microbial ecology in aquaculture systems, such as knowing the types, numbers, and sources of bacteria commonly associated with different developmental stages, could be useful for manipulating microbiota as a strategy to prevent pathogenic infection or to improve nutrition (Deven et al. 2009).

The intestinal microbiota has important and specific metabolic, trophic, and protective functions. The normal (resident) microbiota of the gut confers many benefits to the intestinal physiology of the host. Some of these benefits include the metabolism of nutrients, contribution of the colonization resistance, antagonistic activity against pathogens, immunomodulation and etc. The intestinal microbiota has a profound impact on the anatomical, physiological and immunological development of the host (Rawls et al. 2004). Thus, establishing a healthy microbiota plays an important role in the generation of immuno-physiologic regulation by providing crucial signals for the development and maintenance of the immune system. Understanding how the fish immune system generally responds to gut microbiota may be an important basis for targeting manipulation of the microbial composition. This might be of special interest to design adequate strategies for fish disease prevention and treatment. The intestinal microbiota possesses antagonistic activity against many fish pathogens and participates in infection-protective reactions. Yoshimizu & Ezura (1999) reported that fish intestinal bacteria such as *Aeromonas* and *Vibrio* spp. produced antiviral substances. The bacterial flora of the GI tract of fishes in general, represents a very important and diversified enzymatic potential. It is capable of producing proteolytic, amylolytic, cellulolytic, lipolytic, and chitinolytic enzymes, which is important for digestion of proteins, carbohydrates, cellulose, lipids and chitin. The enzyme producing microbiota can be beneficially used as probiotic supplements while formulating the fish diet, especially in the larval stages. It presents a scope for fish nutritionists to use the enzyme producing isolates as a probiotic in formulating cost-effective fish diets. However, much more research should be conducted to determine if the addition of such isolates to fish feeds do, in fact, provide some kind of benefit to the fish involved before advocating their use.

Micrococcus luteus, *P. fluorescence* and *S. putriefaciens* were the prevalent bacteria species within all the bacterium analyzed in the tested samples (Table 1), which partly reflected that them was the main bacteria species in the intestine of striped catfish. Sakata et al. (1984) observed that *Vibrio*, *Aeromonas* and *Pseudomonas* were the predominant bacterial genera in tilapia intestine. Sugita et al. (1982) found that the gut of tilapia contained a wide variety of bacterial species with the predominant genera being *Pseudomonas* and *Aeromonas* species. In fact, the microflora structure in the intestine of striped catfish was affected by different species, aquaculture environment, the diet, original hatching farm that the fry came from, and etc. In present paper the bacteria such as *A. facalis* and *B. cereus* was the minor percent ones in the intestine of striped catfish (Table 1), which *A. facalis* was only found in control group, that show this bacteria in the intestine of striped catfish without additive nucleotide increase amino acid. With the increase of dietary nucleotides, the bacteria displayed the increasing trends until 0.5 treatment, this is in agreement with other reports of livestock, however, the harmful bacteria *A. hydrophila* was detected in the tested intestinal samples (Table 1) in 1 and 0.75% treatment was less than other groups. Mahious (2006) also reported that Oligofructose cannot improve total SCFA and lactate content of the spiral valve, this might reflect the complication of the micro-ecology in the digestive tract of hybrid tilapia. The benefit bacteria, *C. urealyticum* and *A. faecalis* that produce amino acid and nucleotide was observed in fish fed dietary contained 0.25% nucleotide and control group that showed dietary consist a little or without nucleotide need bacteria to increase nucleotide. As a whole, fish fed the highest amount of nucleotide contained minimum levels of intestinal microbiota.

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